Variable Pathlength Spectroscopy for the Determination of Protein Extinction Spectra and Protein Quantitation

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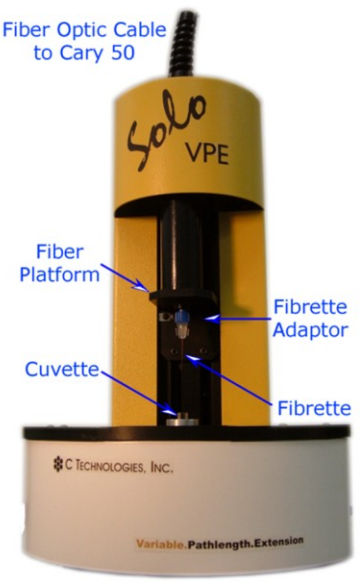
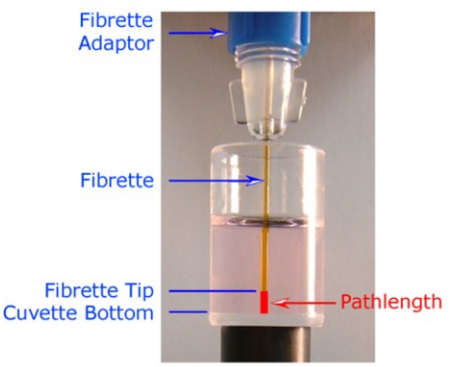
Spectroscopy in various forms are important tools for the structural and functional characterization of biomolecules. Simple absorption spectroscopy is perhaps most familiar from its use in the quantification of biomolecules by both direct and indirect methods. The latter assays are well known and utilize various chemical reactions to provide a signal that is quantified by spectroscopy. These methods generally require the use of a standard which is often used to construct a standard curve. It is assumed that the chemical reactivity of the standard mirrors that the molecule of interest. However, if an extinction coefficient for the molecule is known, then a absorbance reading leads directly to the concentration without the use of any standard. Several authors have elaborated on methods to determine the extinction coefficient of proteins containing tryptophan and tyrosine with a small contribution from disulfides. The work demonstrates the use of variable pathlength spectroscopy using the SoloVPE, for quantitation and the determination of extinction spectra.

Introduction to the SoloVPE



The SoloVPE operates in conjunction with a Cary 50 spectrophotometer. The Cary provides spectrally separated light to one end of a fiber optic cable which has its other end attached to a fiber platform in the SoloVPE. At the platform, the light is delivered to one end of a disposable "Fibrette" (a single piece of optical fiber) attached to the fiber platform by the Fibrette adaptor. The other end of this Fibrette is submerged within the sample in a cuvette located below the fiber platform. Light emerging from this submerged tip, passes through the sample to a detector located beneath the cuvette. The detector returns an electrical signal to the Cary 50 proportional to the light intensity impinging on it for further processing into an absorbance signal.



Pathlength for the SoloVPE is defined by the distance from the submerged tip of the Fibrette to the bottom, inside surface of the cuvette. The depth of submersion is controlled by the position of the fiber platform to which the Fibrette is attached via the Fibrette Adaptor. The vertical position of the fiber platform is itself determined by a software driven stepper motor; thereby permitting precise changes in the positioning of the Fibrette tip within the sample. This control places the pathlength used for any given measurement at the complete discretion of the user.

The author wishes to thank C-Technologies, Inc for providing access to the instrument used in these studies. Further information on The SoloVPE can be found at http://www.solovpe.com/

The foundation of quantitative spectroscopy is

$$\text{Beer's Law} \qquad A = E\,c\,l$$

where A is the absorbance of a solution through a pathlength, l, containing molecules at concentration, c. E is a property of the molecule, its extinction coefficient, and has units the reciprocal of c and l. This relationship is linear in both c and l. However, deviations from linearity are well known, and may arise from instrumental limitations or changes in molecular properties in solution as c increases. The SoloVPE permits the quick measurement of complete spectra at multiple pathlengths (pathlength scanning) using a SINGLE sample; from which one constructs a "section plot" of absorbance vs pathlength. The slope (m) in a section plot is Ec, so that knowledge of either permits the other's calculation.

$$m = d(A)/d(l) = E\,c$$

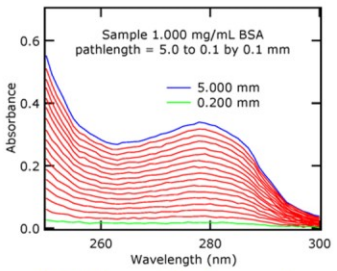
The slope measurement provides redundant information over what would be obtained at a single pathlength, improving accuracy. Moreover, one avoids the need for dilution should the concentration of the initial stock be in a non-Beer's law region for the instrument of choice.

One of the simplest applications of slope based spectroscopy provides a measurement of the extent of dilution in this case we measure slopes on the stock solution and on the diluted material such that

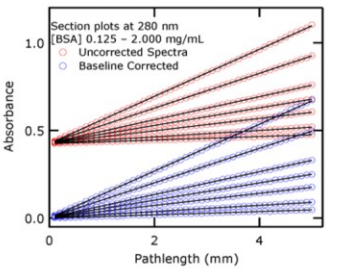
$$m_{Stock}/m_{Diluted} = E[Stock]/E[Diluted] = [Stock]/[Diluted]$$

one has a quick measure of the dilution ratio and if the stock concentration is known then the concentration in the diluted material is readily determined. This can be particularly useful when one is diluting highly concentrated stock solutions, where the volume measured by the typically used air displacement pipette may be inaccurate for a variety of reasons including that the pipette was not calibrated to measure high viscosity samples.

Relationship between Slope and Concentration

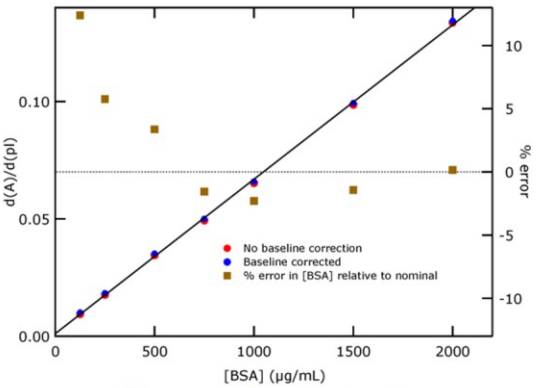


Pathlength Scanning



Extracted Section Plots

If the buffer has no significant absorbance as a function of pathlength, then one can work with non-baseline corrected data, and the measured absorbances will be offset by an instrumental baseline. The Section plots shown were extracted from both baseline corrected and uncorrected spectra. While not shown, intercepts were independent of concentration for both sets. This shows that one does NOT need to do buffer baselines in this case, i.e. the buffer has no significant pathlength dependent absorbance.



Slope vs Concentration

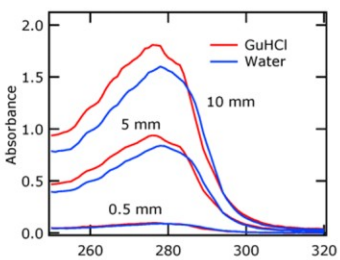
The slopes at each nominal concentration are the same with and without baseline correction. Also shown is the relative error when the slope was converted to a concentration

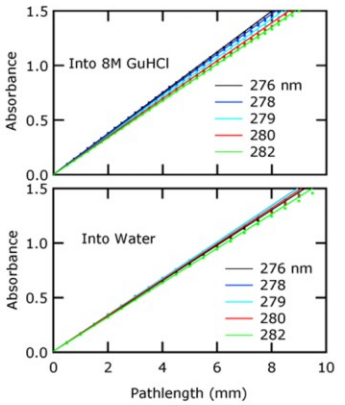
Extinction Coefficients & Spectra

For quantitation, an extinction coefficient must be known. If the material could be carefully weighed, then a section plot would be used to measure the extinction coefficient for future use. For most biologicals, dry weights are not easily measured. For proteins, the dominant contributors to absorbance at 280 nm are the tryptophan (W) and tyrosine (Y) residues with a minor contribution from the disulfide bonds (CC). Generally we know the complete sequence of proteins, although the disulfide bonding pattern may not be fully characterized, thus we have an accurate accounting of the major contributors to the the absorbance per polypeptide chain. Furthermore, most proteins fully denature in 6 M Guanidine HCl (GuHCl) so that environmental effects arising from the folded protein can be eliminated. Edelhoch, originally developed a method exploiting these facts to measure W and Y content of proteins by determining the extinction coefficients for W, Y and CC in 6 M GuHCl. Here this approach coupled to the slope measurements made possible by the SoloVPE. The concept is that an identical aliquot of protein in a given buffer, is diluted into the same volume of either buffer or GuHCl solution. Thus the ratio of slopes measured in the two solutions is the ratio of their extinction coefficients..

$$m_{water}/m_{GuHCl} = E_{water}[P]/E_{GuHCl}[P] = E_{water}/E_{GuHCl}$$

Moreover, E in GuHCl is readily calculated at select wavelengths for the protein in GuHCl given the amino acid sequence, knowledge of disulfide bonding (BSA W=2, Y=20, CC=17) and tabulated values for the residues as measured in GuHCl (See table for BSA results). This extinction coefficient, in turn, can be used to calculate the concentration if an aliquot of sample is accurately diluted into GuHCl and the slope of a section plot determined. The commercial material used in this study had a reported concentration of 275 mg/mL. Using the 280 nm section plot, a known dilution of 50 µL into 5.05 mL, and the calculated extinction coefficient (3.9 x 10^4/M/cm, see table), the value was determined to be 299 mg/mL a value about 8% high.





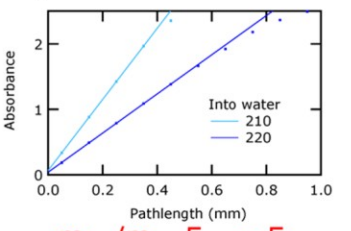
After E_{GuHCl} is calculated and section plots in water and GuHCl are measured, the computation of the E_{water} is straightforward. The table shows the values computed in this study.

$$m_{water}/m_{GuHCl}\ E^{calc}_{GuHCl} = E_{water}$$

	GuHCl Calculated	Water Measured
210		121.90
220		67.17
276	4.23	3.66
278	4.14	3.75
279	4.03	3.75
280	3.90	3.68
282	3.69	3.42

Extinction coefficients $M^{-1}cm^{-1}$ x10^{-4}

The SoloVPE allows different pathlength increments in different spectral regions. Thus good section plots can be created in regions with disparate extinction coefficients, such as the near and far UV regions, in a single run.



$$m_{220}/m_{280}\ E_{280} = E_{220}$$

Using this approach complete extinction spectra, with a single sample.

Edelhoch, H. (1967). "Spectroscopic determination of tryptophan and tyrosine in proteins." Biochemistry 6: 1948-1954
Gill, S. C. & P. H. van Hippel (1989). "Calculation of protein extinction coefficients from amino acid sequence data." Anal. Biochemistry 182: 319-326.
Pace, C. N., et al. (1995). "How to measure and predict the molar extinction coefficient of a protein." Protein Science 4: 2411-2423.

The slopes from section plots measured by the SoloVPE provide a rapid means to quantify dilution ratios. When combined with an accurate measurement of concentration, through perhaps accurate weighing, or quantitative amino acid analysis for proteins provides a facile, direct means to determine an extinction coefficient. Alternatively the approach described above for proteins, leads to extinction coefficients without knowledge of the concentration; moreover, the data can be used to construct complete extinction spectra. Pathlength scanning and the created section plots whose slopes are used in these calculations, provides redundancy that reduces error over that provided by a single aborption measurement. Moreover these measurements use a single sample so that dilutions are not needed and the material can be used for other purposes.

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